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Examination of Neutrophil Function in a Rat Model of Decreased Host Resistance Following Burn Trauma

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The high incidence of serious opportunistic infection following human burn injury has been well documented. Investigations of the mechanisms of this acquired susceptibility have demonstrated several defects in phagocytic defenses. An established rat burn infection model was modified for study of neutrophil function in animals with a 60% burn injury. These 350-g rats received a 35-ml saline resuscitation, and when not further stressed, 80% of the animals survived to healing. Burned animals were found to have decreased inflammatory responses to intraperitoneal injections of heat-killed *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and sterile sodium caseinate. These reductions could not be explained by neutropenia. Prior immunization with heat-killed *Pseudomonas* did not improve the inflammatory response to homologous organisms injected intraperitoneally, but levamisole treatment did improve the imflammatory response. Epinephrine injection (intravenous) showed that burned animals have a markedly reduced proportion of marginated neutrophils but an increase in total peripheral neutrophil counts. The stress hormones corticosterone and catecholamines were elevated during times of decreased inflammatory responsiveness; additionally, neutrophils from burned animals had decreased adherence to nylon fiber. Serum from burned animals decreased in vitro adherence and chemotaxis of purified normal rat neutrophils.

Susceptibility to *Pseudomonas aeruginosa* injection appears to be primarily a host-dependent variable; that is, pseudomonas infection in humans is most frequently associated with, or perhaps a symptom of, underlying immunosuppression. The association of pseudomonas infection with burn injury is a frequently quoted example. Although pseudomonas infection of burn wounds has become less frequent [1, 2], it remains a good prototype of opportunistic infection.

The immunologic basis for susceptibility to opportunistic infection associated with burn trauma has been the subject of numerous investigations. Patients with large burns frequently display immunologic defects [3]. The PMN has been the most intensively studied host defense system. Following severe injury, in vitro neutrophil defects have been found to include altered chemotaxis [4–7], decreased microbicidal activity [8–10], decreased lysosomal enzyme content [7, 11], decreased oxygen consumption [12], decreased nitroblue tetrazolium reduction [13], decreased chemiluminescence [14], altered NADH-NADPH oxidase activity [15], and decreased glucose oxidation [16]. Additionally, decreased inflammatory responsiveness as measured by skin windows has been described [17].

An established rat burn model of susceptibility to *Pseudomonas* has been examined for possible underlying abnormalities. The Walker-Mason rat burn model was used [18, 19]; this model has been used extensively at this institute and elsewhere to investigate antimicrobial chemotherapies [20], vaccines [18], and trauma-associated metabolic changes [21]. These investigations indicate that *Pseudomonas*-infected animals die, and noninfected burned rats show maximal stress-related altered metabolism early in the second week after injury.

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In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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Materials and Methods

Rat burn model. Adult, male, 340–360-g Sprague-Dawley rats were anesthetized with sodium pentobarbital (1 mg/40 g); the dorsal and ventral surfaces were clipped with a number 40 Oster blade. A 30% full-thickness scald injury [19]

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was first inflicted on the dorsum by immersion for 10 sec in boiling water. The animals were then removed from the burn mold and injected ip with 35 ml of normal saline; they were next returned in the prone position to the mold and a 30% ventral burn was inflicted by immersion in boiling water for 3 sec. After burning, the animals were individually caged and allowed to eat and drink water ad libitum in a controlled-temperature (20–25 C) environment with controlled lighting.

Examination of leukocyte count patterns in peripheral blood. Total and differential WBC counts were performed in a group of six animals before burning and daily for the first 10 days postburn. Four animals were sham burned and bled daily as controls. Blood was obtained from unanesthetized animals by tail venipuncture. Total WBC counts were determined with a Coulter counter (Model ZBT), and differential cell counts were made on smears stained with Wright-Giemsa stain.

Examination of ip inflammatory responses. Burned animals were examined for their ability to muster a neutrophilic exudate response following ip injection of leukoattractants. Sterile casein [22], heat-killed *P. aeruginosa*, or heat-killed *Staphylococcus aureus* were used. The peritoneal challenge inocula were 10 ml of sterile 10% sodium caseinate, 10 ml of saline-suspended, heat-killed *P. aeruginosa* (10^8 organisms/ml), or 10 ml of saline-suspended, heat-killed *S. aureus* (10^7 organisms/ml). The response to *P. aeruginosa* was examined in animals injected with heat-killed organisms 30 days and 10 days before burning. These animals were also examined for agglutinating antibodies at the time of ip injection. Burned control animals were used to measure total hemolytic complement [23] activity at the times of ip challenge. Unburned, injected animals were used as controls.

Exudates were examined (18–24 hr after the ip injection) by injecting 60 ml of heparinized saline (10 units of heparin/ml) into the peritoneum of rats anesthetized with Penthrane® (Abbott Laboratories, North Chicago, Ill.). The peritoneum was opened by midline laparotomy, and the exudate was collected into graduated cylinders. The peritoneal cavities were liberally washed with heparinized saline, and the total recovered volume was measured. Total number of cells recovered and differential cell counts were determined.

Circulating neutrophil response to epinephrine given iv. Total peripheral neutrophil counts (circulating plus marginating) [24] were measured from rats anesthetized with sodium pentobarbital. Blood samples were drawn via a cannula placed in the abdominal aorta before iv injection of epinephrine into the penile vein. Epinephrine (0.1 ml) was used at a 1:10,000 dilution [24]. Burned animals were examined on the ninth day postburn. Unburned animals were used as controls.

Circulating neutrophil adherence to nylon fiber. Neutrophil adherence to nylon fiber was measured by a modification of the method of MacGregor et al. [25]. Heparinized (10 units/ml) blood was taken from the abdominal aorta immediately after Penthrane® anesthesia. This blood was passed over nylon-fiber columns packed in Pasteur pipettes as originally described. The flow rate was limited by using an iv flow valve placed on a small length of tubing attached to the bottom of the pipettes. Neutrophil counts before and after passing the blood through the column were used to determine the percent adherence. One milliliter of heparinized blood was used per column, and all specimens were examined in triplicate. Experiments were conducted at 37 C with a flow time of 5–7 min. A dose-response curve for adherence of neutrophils from normal rat blood as a function of nylon-fiber weight was used to select column weights (see below).

Corticosterone and catecholamine determinations. Serum corticosterone levels were measured fluorometrically [26]. Total levels of plasma catecholamines were determined by the Cat-A-Kit® radioenzymatic assay (Upjohn, Kalamazoo, Mich.). Blood samples were taken from the abdominal aorta immediately after Penthrane® anesthesia. Total 24-hr urinary output of catecholamines was determined in animals housed in metabolic cages, with the use of the Oxford® fluorometric catecholamine assay (Oxford Laboratories, Foster City, Calif.) These animals were conditioned for 1 week before burning.

Levamisole treatment. The effect of levamisole [27] on the peritoneal response to heat-killed *P. aeruginosa* was examined. The drug was infused continuously, with the use of the Alzet® 1702 implantable minipump (Alza Corp., Palo Alto, Calif.), at doses of 1 mg/kg or 10 mg/kg per day [28]. Saline-loaded pumps were used to infuse control animals. Nonburned and burned, untreated animals were used as additional controls. The pumps were implanted subcutaneously over the

skull immediately after burning. Challenge with *P. aeruginosa* was done as described above.

Isolation of rat neutrophils from peripheral blood. Initial attempts to isolate rat leukocytes by dextran-sedimentation techniques used for humans and other species were unsuccessful. It was therefore necessary to devise an alternate method for isolation of rat neutrophils. Rat RBCs were found to sediment when whole blood was mixed with gelatin. A solution of 3% gelatin (USP), 0.7% NaCl, and 0.2% $CaCl \cdot 2H_2O$ was found to be optimal for separation of RBCs and neutrophils.

For isolation, two volumes of heparinized blood was mixed with one volume of gelatin solution and allowed to settle at $1 \times g$ for 30–40 min. After sedimentation the supernatant was centrifuged at 150 $\times$ g for 10 min, and the pellet was exposed to 10 ml of 0.87% NH_4Cl until red cell lysis had progressed to the stage where the suspension was transparent ($\sim$10 min). The tube was then centrifuged for 10 min at $150 \times g$. After centrifugation the pellet was resuspended in Hanks' balanced salt solution (HBSS) and layered onto Ficoll-Paque (Pharmacia, Piscataway, NJ) at a cell suspension to Ficoll-Paque ratio of 4:1. This preparation was then centrifuged at room temperature for 40 min at $400 \times g$. After centrifugation the pellet was recovered and resuspended in HBSS, and the cell preparation was placed in an ice bath. Cell counts and differential counts were then performed. The cell suspensions were kept in ice for 30 min before any further procedures were conducted.

Effect of serum from burned rats on the adherence of normal rat neutrophils. Normal rat peripheral neutrophils were prepared for each experiment from five rats as described above. One milliliter volumes of pooled PMNs (10^7 cells/ml) were mixed with an equal volume of normal serum or serum collected on the third or ninth day postburn. The serum-cell mixtures were incubated at 37 C for 30 min, diluted 1:4 with HBSS, and assayed for adherence on the 50-mg nylon-fiber columns. Control experiments were conducted in which diluted sera were passed over the column before the cells were added. This was done to test the possibility that protein differences in the sera might have different charge-blocking effects on the nylon fibers and thus alter neutrophil adherence.

Effect of serum from burned rats on the chemotaxis of neutrophils from normal rats. Normal rat neutrophils were prepared by the procedure described above. The chemotactic effect of casein (MCB Chemicals, Norwood, Ohio) on neutrophils from normal rats was examined after incubation of the neutrophils with normal rat serum, serum collected three days postburn, or serum collected nine days postburn. The cell-serum incubation protocols were the same as those outlined for neutrophil adherence. A modified Boyden Chamber was used to measure chemotaxis [4]. Millipore filters (3 μm) were used to separate the lower and upper wells of the chamber. Casein (5 mg/ml in HBSS) was added to the lower chamber until the filter was wet. The upper chamber was next loaded with 0.5 ml of the serum-cell mixtures and incubated at 37 C for 1 hr. Control chambers, for determination of the random migration of neutrophils, contained HBSS in the lower chamber. After incubation, the filters were removed from the chambers and stained with hematoxylin and eosin, cleared with xylene, mounted on microscope slides, and covered with coverslips. Cell migrations were measured by the leading front assay, which measures the depth of cell migration into the millipore filter [29]. The filters were examined with the use of a 50$\times$ oil-immersion objective by focusing into the filter until one or two cell nuclei were in focus. The fine-focus micrometer reading on the microscope was recorded, and the focus was then moved until the top surface of the filter was in focus. The focal distance between the front edge and the top of the filter was read directly from the micrometer. Multiple measurements for each filter were made, and chemotaxis was determined by subtracting the random-movement distances of control chambers, without casein, from the chemotaxis observed in the casein-containing chambers.

Statistical analyses. Data were compared by analysis of variance or Student's *t* test. Control and experimental animals in each experiment were from the same shipment lot.

Results

When not further stressed, 350-g rats with burns on 60% of their skin had greater than 80% survival at 30 days postburn. The majority of deaths occurred within 48 hr postburn. Animals with spontaneous, culture-proven sepsis were excluded from these studies.

Burned animals showed a temporal pattern of peripheral leukocyte numbers (figure 1) similar to that reported for burned humans [30]. An initial leukocytosis was followed by a moderate depression that was succeeded by a second elevation persisting for the period studied. Differential count-

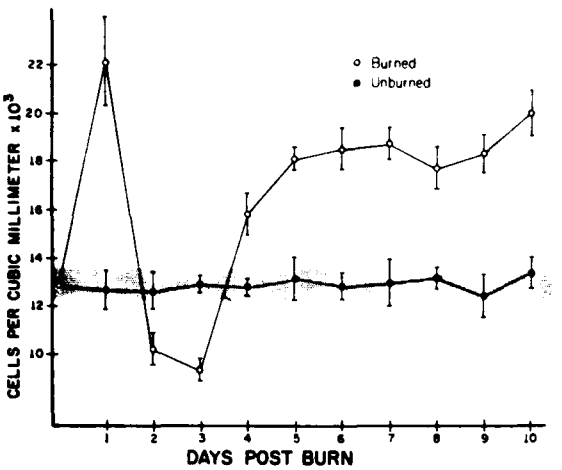

Figure 1. Temporal effect of 60% burn injury of rats on total white blood cell count. Shaded area represents the mean cell counts (± 2 SE) obtained from pooled, preburn blood.

ing showed that neutrophils were principally responsible for the observed elevations (figure 2). Lymphocyte counts showed a significant lymphopenia on days 1 through 3 (figure 3). Lymphocyte counts were within the preburn values by day 4 and thereafter remained at that level. Immature cells (metamyelocytes) constituted <10% of the granulocytes counted during the observation period. At no time did the absolute neutrophil count drop below the preburn values.

Examination of peritoneal inflammatory responses following ip injection of casein or *S. aureus* showed significant reductions of neutrophil accumulation on day 3 and day 9 postburn (figures 4 and 5). Comparison of circulating neutrophil counts following casein injection showed burned animals to have equal or greater counts than did injected unburned controls.

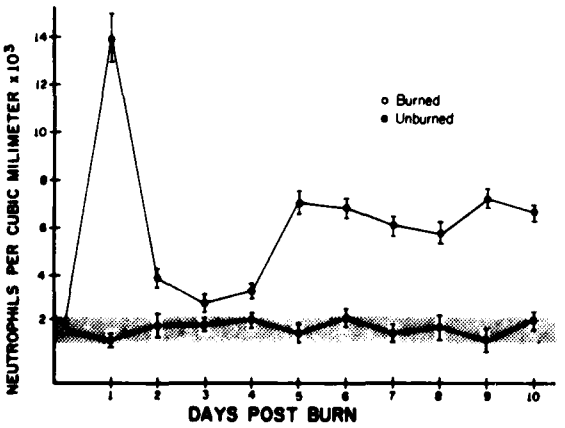

Figure 2. Temporal effect of 60% burn injury of rats on the absolute neutrophil count. Shaded area represents the mean cell counts (± 2 SE) obtained from pooled, preburn blood.

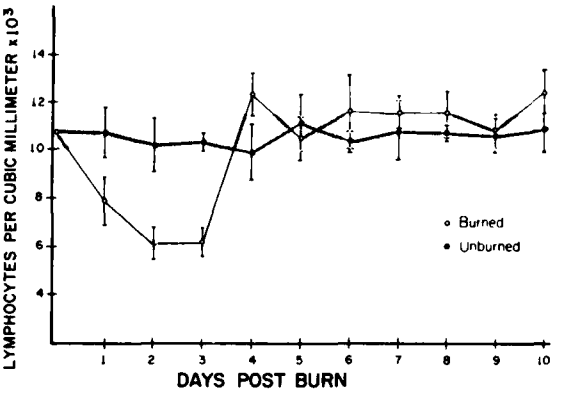

Figure 3. Temporal effect of 60% burn injury of rats on the absolute lymphocyte count. Shaded area represents the mean cell counts (± 2 SE) obtained from pooled, preburn blood.

Despite preburn exposure to homologous heat-killed *P. aeruginosa*, burned animals had a significantly depressed peritoneal response (figure 6). Unburned and burned rats had equal antibody responses; the reciprocal geometric mean titers ± SE were 84.4 ± 1.28 and 97 ± 1.36, respectively.

Measurement of total hemolytic complement (C'50) in three groups of five rats showed no significant differences among unburned rats and rats at days 3 and 9 postburn. Values were 37.15 ± 2.84, 44.98 ± 5, and 40.90 ± 3.88, respectively. The human control C'50 value was 32.

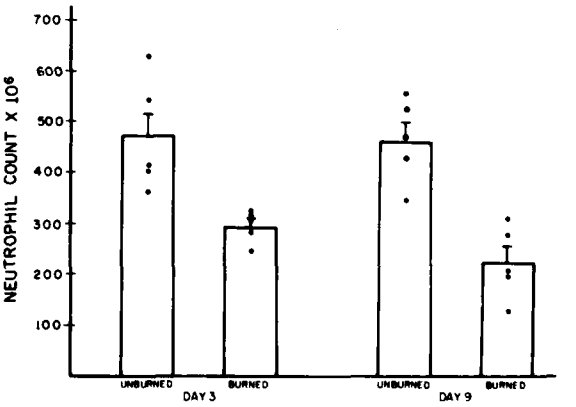

Figure 4. Recovery of peritoneal neutrophils after ip injection of casein into normal or 60% burned rats. Neutrophils were collected from animals on the third and ninth days postburn.

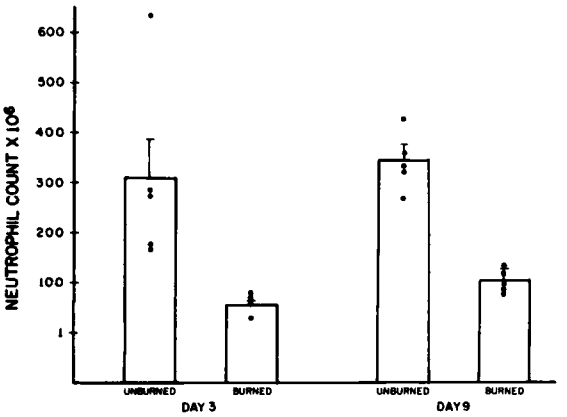

Figure 5. Recovery of peritoneal neutrophils after ip injection of *Staphylococcus aureus* into unburned or 60% burned rats. Neutrophils were collected from animals on the third and ninth days postburn.

The circulating neutrophil response of an anesthetized normal rat to epinephrine injection is shown in figure 7. There was a rapid rise in the absolute neutrophil count, which returned to normal by ~10 min postinjection. RBC counts taken from the same specimen showed no significant elevation; the neutrophil increase does not represent hemoconcentration. The ratio of the postinjection neutrophil count to the preinjection value was taken as the proportion of the total number of peripheral neutrophils that were marginated. The peak neutrophil response of normal rats and rats burned nine days earlier is shown in table 1.

Burned animals showed an elevated preinjection neutrophil count ($P < .01$). Both burned and normal rats showed neutrophil elevations postinjection ($P < .01$). Burned animals had a total peripheral neutrophil count larger than that of control animals ($P < .05$); however, burned rats had a markedly reduced proportion of marginating cells, as reflected by the difference in the percent increase between the two populations ($P < .001$).

There have been several reports of decreased adherence of neutrophils to nylon fiber following demargination [31, 32]. Neutrophil adherence was therefore investigated with cells from the 60% burned rat. The dose-response relationship of normal rat neutrophil adherence to various weights of nylon fiber is presented in figure 8. Pooled blood from three normal rats was used in the experiment. A weight of 80 mg was selected for comparing the adherence of cells from burned and normal rats. Burned rat and normal rat neutrophil adherence was compared on day 9 postburn. As shown in figure 9, neutrophils from burned rats had significantly reduced adherence to nylon fiber. The fact that nylon-fiber adherence has been reported to mimic endothelial cell adherence suggests an in vitro correlate to the decreased in vivo margination described above [32].

Levels of serum corticosterone were measured in groups of five rats on the third and the ninth day postburn. Control rats had a mean level ± SE of 27.31 ± 2.07 μg/100 ml. Burned rats showed levels of 51.85 ± 2.30 μg/100 ml on day 3 postburn and 33.31 ± 1.18 μg/100 ml on day 9. Analysis of variance (Scheffé) showed that the elevation on day 3 postburn was significant ($P < .001$). Day 9 values, though suggesting elevation, were not significantly different from those of control animals.

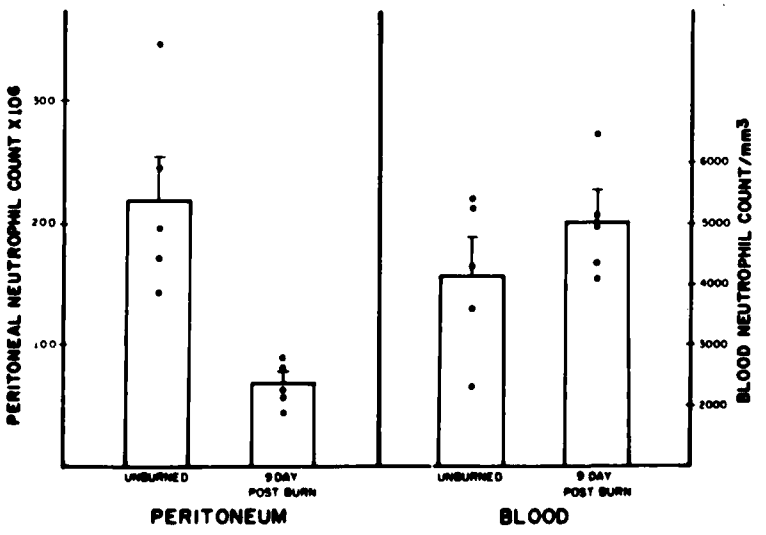

Figure 6. Recovery of blood and peritoneal neutrophils after ip injection of *Pseudomonas aeruginosa* into normal or 60% burned rats. *P. aeruginosa* was injected on day 9 postburn and neutrophils were collected on day 10 postburn.

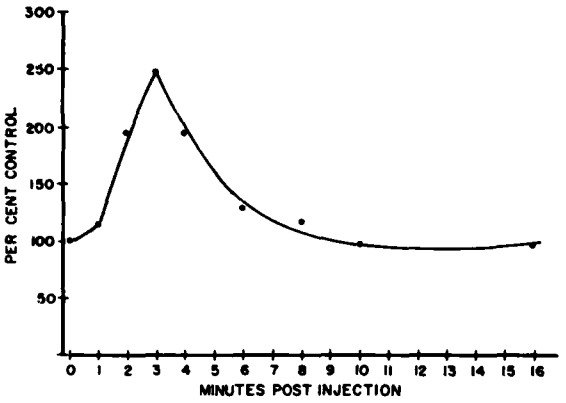

Figure 7. Effect of iv-injected epinephrine (0.1 ml of a 1:10,000 dilution) on the absolute neutrophil cell count in an anesthetized 350-g rat.

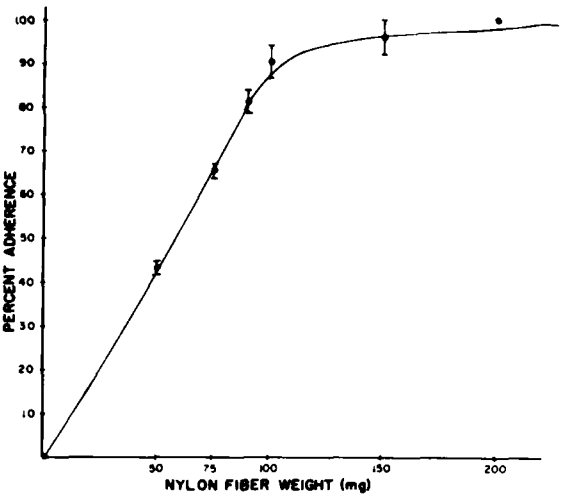

Figure 8. Dose-response relationship of normal rat neutrophil adherence to increasing weights of nylon fiber.

In separate experiments, day 9 postburn rats had markedly elevated plasma and urinary catecholamine levels ($P < .01$). Urine values ± SE (six rats per group) were 0.363 ± 0.104 μg/day for controls and 2.27 ± 0.218 μg/day for burned rats. Plasma levels (eight rats per group) were 8,235 ± 259 pg/ml for controls and 16,634 ± 2,578 pg/ml for experimental animals.

Continual infusion of levamisole significantly increased the inflammatory response to *P. aeruginosa* given ip to burned rats (figure 10). At both doses, treated animals had significantly elevated cell counts when compared with burned or burned and sham-treated animals ($P < .01$). Levamisole treatment did not, however, return the response levels to those of unburned animals.

The effect on adherence of incubation of isolated normal rat neutrophils with serum from burned rats is presented in table 2. Serum from either day 3 or day 9 postburn rats decreased the neutrophil adherence to nylon fibers. The effect of serum from burned rats on the adherence of normal neutrophils is similar to the effect of serum taken from demarginated humans on normal neu-

Table 1. Neutrophil response of anesthetized normal or burned rats to epinephrine administered iv.

Group	Preinjection neutrophil count/mm³	Postinjection neutrophil count/mm³	Percent increase
Normal	2,014	4,988	148
	1,040	3,025	190
	1,630	3,915	140
	1,206	3,350	177
	1,936	4,290	122
Mean	1,565	3,914	155
Burned*			
	3,131	4,052	29
	4,781	5,474	14
	3,003	4,298	43
	5,161	6,613	28
	3,920	5,368	37
Mean	3,999	5,261	30

NOTE. All rats weighed ~350 g, and there were five rats per group.

* Rats were tested 9 days postburn.

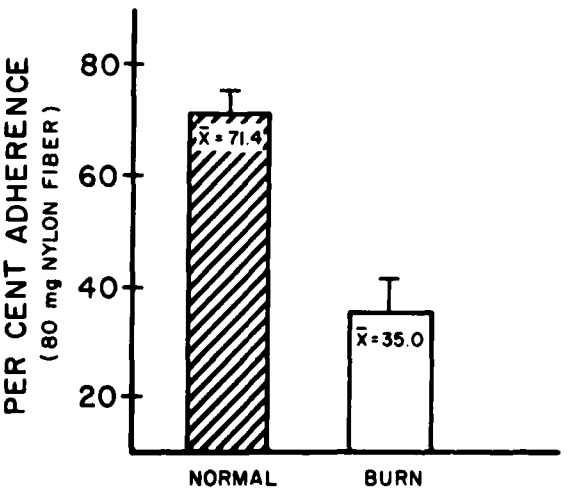

Figure 9. Relative adherence of neutrophils from normal or 60% burned rats to nylon fiber (80 mg). Six animals from each group were tested on the ninth day postburn. The percent adherence of neutrophils from burned rats was significantly lower than that from normal neutrophils ($P < .01$). $\bar{X}$ = mean.

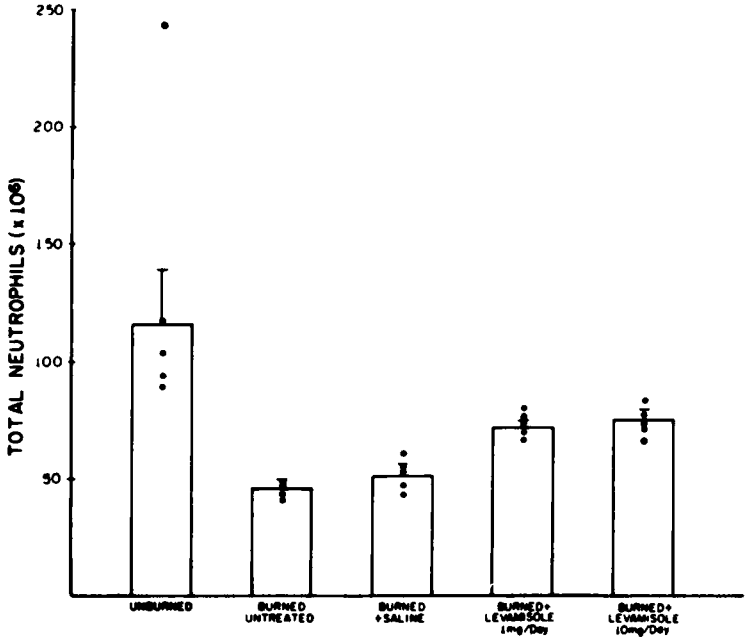

Figure 10. Recovery of peritoneal neutrophils after levamisole treatment of normal or 60% burned rats previously inoculated ip with *Pseudomonas aeruginosa*. Animals were compared on the tenth day postburn.

trophil adherence [31]. Control experiments included passing cells over nylon-fiber columns precoated with normal or burn sera. No difference in effect on adherence was seen between these sera. This confirms an earlier report that nylon-fiber adherence is not the result of activated plasma proteins [33].

The effect on chemotaxis of normal granulocytes after preincubation with sera from rats three days postburn is presented in table 3. The effect on chemotaxis of sera from rats nine days postburn is presented in table 4. In both cases, these sera depressed the chemotaxis of normal granulocytes. A similar inhibition of normal chemotaxis has previously been reported with the use of sera from burned humans [34]. This is the first report in an animal burn model of serum-associated depression of chemotaxis.

Table 2. Effect of serum from burned rats on normal rat neutrophil adherence to nylon fiber.

Experiment no.	Percent adherence after treatment with indicated serum		
	Normal	Day 3 postburn	Day 9 postburn
1	70.6	21.3	36.0
	82.5	31.1	49.0
	62.5	36.4	44.2
	69.9	8.2	62.0
2	62.0	13.4	0
	45.0	33.6	21.2
	39.0	35.0	26.8
	76.0	42.0	40.0
	62.1	51.0	11.9
	62.0	51.0	44.5
3	44.0	33.0	37.9
	64.9	25.8	47.8
	67.4	53.0	55.0
Mean ± SE	62.14 ± 4.1	33.43 ± 3.72*	36.6 ± 4.3*

* $P < .01$.

Discussion

Human and rat burn wound infections with *P. aeruginosa* follow a very similar bacteriologic and histopathologic course [35]. Basically, the infection is a progression of bacterial growth from colonization of nonviable surface tissue to massive accumulation of bacteria in the deeper eschar, subsequent invasion of viable tissue, and eventually, bacteremia. At autopsy, disseminated infections with metastatic lesions of the lungs, kidneys, spleen, liver, and other organs are frequently observed. The similarity between human and experimental rat infection makes the rat burn model an excellent tool for investigating virulence mechanisms of *P. aeruginosa*. Unlike mouse burn models in which bacteria are injected beneath the skin surface to demonstrate proposed virulence factors [36, 37], the rat burn surface model requires expression of microbial aggressive properties needed to invade the host. Virulence factors may be missed

Table 3. Effect of rat serum obtained three days post-burn on the chemotaxis of normal rat neutrophils.

	Distance migrated (μm) by neutrophils after treatment with indicated serum	
Experiment no.	Normal	Burned-rat
1	55	40
	92	50
	85	44
	80	62
	81	78
	82	60
	85	58
	98	63
	108	61
2	90	50
	99	68
	90	44
	85	65
	72	67
	97	70
	105	61
	90	62
	88	57
Mean ± SE	87.3 ± 3.87	58.8 ± 2.58*

NOTE. Each number is one measurement in the experiment.
* $P < .01$.

or misinterpreted by injecting characterized strains beneath the burn surface [38].

The intent of this investigation was to use the rat model to examine the other side of the burn-infection coin. The noninfected, burned rat has decreased peritoneal inflammatory capacity. This finding could not be explained by decreased numbers of circulating cells or decreased complement activity. The presence of antibody did not normalize the response to killed *P. aeruginosa* injected ip. Burned rats had a decreased proportion of marginated neutrophils and decreased adherence of neutrophil to nylon fiber. This relationship is similar to that seen when demarginated human blood is examined for neutrophil adherence to nylon fiber [39]. The elevation in the levels of examined hormones of the burned rat may be responsible for the observed altered adherence. The partially restorative effect of levamisole supports this concept since levamisole is known to reverse selected neutrophil dysfunctions that follow exposure to trauma-related hormones [40, 41]. The findings that burned-rat sera decrease in vitro neutrophil adherence and chemotaxis also suggest that humoral factors are at least in part responsible for the observed decreased neutrophil responsiveness. If one assumes inflammation to be an important component in host defense against *P. aeruginosa*, the burned rat would predictably be a susceptible host.

The metabolic alterations that follow burn injury are the most extreme seen in any hospitalized population [42]. Burned patients demonstrate hypermetabolic, hypercatabolic, and hyperhemodynamic adaptations to burn stress [42–44]. These responses are related to the burn size. Wilmore and others have explained that the metabolic adaptation is the result of elevations in levels of catecholamines and corticosteroids [45, 46]. It is interesting to consider in concert the dose-response curves of hypermetabolism related to burn size [45] and decreased probability of survival [47] and relate such to the concept that there are limits to survivable stress as proposed by Selye [48]. Perhaps infection is a symptom of burn disease rather than the most easily explainable cause of death.

Table 4. Effect of rat serum obtained nine days post-burn on the chemotaxis of normal rat neutrophils.

	Distance migrated (μm) by neutrophils after treatment with indicated serum	
Experiment no.	Normal	Burned-rat
1	83	54
	60	30
	54	57
	55	45
	50	90
	67	83
	65	50
	70	60
	50	34
	110	42
	64	35
	54	67
2	85	75
	75	70
	60	45
	75	64
	80	47
	77	70
	65	64
	64	57
	52	38
	71	74
	61	40
	51	82
Mean ± SE	66.58 ± 2.80	57.2 ± 3.38*

NOTE. Each number is one measurement in the experiment.
* $P < .04$.

During the several years that separated presentation and publication of this symposium, numerous papers that relate neutrophil ligand interactions with alterations of in vitro and in vivo function have been published. The phenomenon of selective down-regulation of neutrophils by activated complement [49] and the demonstration of acute complement activation with thermal injury in the rat [50] offer a particularly interesting perspective of the present findings. Although alteration of total complement activity was not observed in this study, the activity of specific components was not investigated. These measurements must be completed to define the role of complement in the observed neutrophil hypoactivity. With the chronic nature of burn wounds and their predictable contamination, it seems quite possible that localized activation of complement or absorption of bacterial products and production of neutrophil-activating factors by the burn wound could exist until healing. The systemic consequence of this chronic wound effect may well be the hyporesponsiveness of neutrophils following burn trauma. It is hoped that the rat burn model of infection may be used further to test this possibility and evaluate the ability of immunomodulatory therapies to improve resistance in the chronically stressed host.

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